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Review

Forty years of Mitchell's proton circuit: From little grey books to little grey cells

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ABSTRACT

It is more than forty years since Peter Mitchell published his first 'little grey book' laying out his chemiosmotic hypothesis. Although ideas about the molecular mechanisms of the proton pumps have evolved considerably since then, his concept of 'coupling through proton circuits' remains remarkably prescient, and has provided the inspiration for the research careers of this author and many others. This review is a personal account of how the proton circuit has been followed from the little grey book, via brown fat and calcium transport to investigations into the life and death of neurons, Hercule Poirot's 'little grey cells'.

The problems encountered by Peter Mitchell in publishing his chemiosmotic hypothesis led him to produce two, privately published, 'little grey books' [1,2]. On p119 of the first of these there stands a figure whose importance is difficult to exaggerate. It is entitled ' the proton circuit' and is reproduced in Fig. 1. While Mitchell's distinctive nomenclature may require some updating (thus the h/d, hydro/dehydro, system corresponds to the ATP synthase) it is a testament to Mitchell's vision that all the key components of the linked ion circuits across the inner mitochondrial membrane have been identified and confirmed – in most cases both functionally and structurally, although a few stubborn components, such as the mitochondrial Ca^{2+} uniporter – remain to be identified at the molecular level.

The proton circuit has been the common strand of research in our group for almost 40 years, and the brief and deliberately introspective review that follows is an attempt to follow the development of ideas – from early studies quantifying the proton circuit in liver [3] and brown adipose tissue mitochondria [4], via investigations into the kinetics of the mitochondrial Ca^{2+} circuit that led to the concept of the mitochondrion as a regulator of cytoplasmic free Ca^{2+} concentration [5], to studies of the proton circuit in intact neural systems – initially isolated nerve terminals (synaptosomes) [6] and subsequently cultured neurons [7,8]. Colleagues will I hope forgive me that the broad scope of the review and space constraints means that it is not possible to do justice to the enormous contributions made by other groups to the four fields to be covered here.

1. The brown fat mitochondrial proton circuit and UCP1

By 1972, two groups had made important observations concerning the failure to observe respiratory control in freshly prepared brown adipose tissue mitochondria (BATM) from hamsters or cold-adapted rats that showed that the uncoupled state was not simply due to inappropriate isolation techniques. Rafael et al. [9] showed that the inclusion of albumin to bind endogenous fatty acids together with a purine nucleotide such as GDP allowed respiratory control to be seen. In the same year the group of Lindberg observed a 'carnitine cycle' in which the simple activation and oxidation of endogenous fatty acids in the presence of CoA, carnitine and ATP induced respiratory control [10]. Subsequently these two results were reconciled when it was realized that the ATP was playing the same 'recoupling' role as GDP in the Rafael study.

These were 'pre-chemiosmotic' studies, and no mechanism was proposed to account for these observations. My own contribution began in 1972 when I observed that BATM were abnormally sensitive to osmotic collapse of the matrix, with resultant inhibition of the citric acid cycle and NAD-linked oxidation [11]. In a KCl medium the matrix slowly expanded and respiration was restored. This indicated an abnormal Cl^- permeability, which was confirmed using a passive swelling technique monitoring light scattering. Excitingly, this permeability was dramatically inhibited by GDP [12]. There therefore seemed to be a linkage between the 'uncoupled' state and anion permeability. It is revealing in the context of 1972 that it was several months before it occurred to me to test whether the lack of respiratory control was associated with proton permeability! The passive swelling technique could be used to test this by adding BATM to media such as

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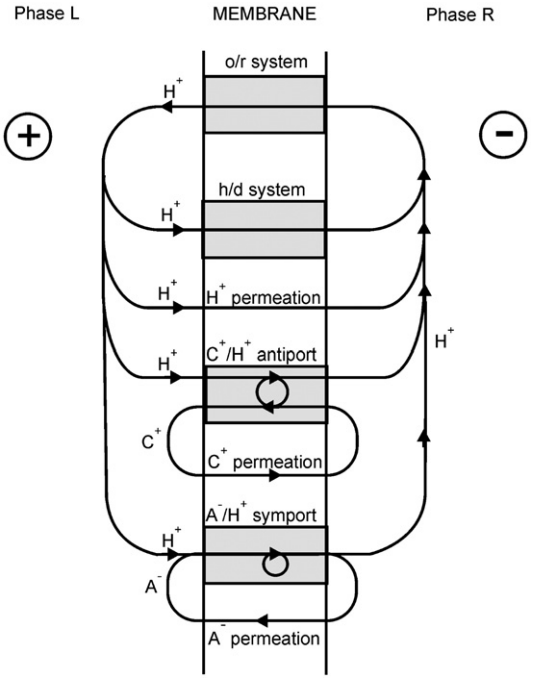

Fig. 1. 'Coupling through proton circuits' from Mitchell's first 'little grey book. The o/r (oxido-reduction) system corresponds to the respiratory chain, the h/d (hydro–dehydro) system to the ATP synthase, H^+-permeation to proton leaks (UCPs), C^+/H^+ e.g. the liver mitochondrial Ca^{2+}/H^+ exchanger, C^+ permeation, e.g. the Ca^{2+} uniporter, while A^-/H^+ symport and A^- permeation describes the action of a classical uncoupler, reproduced from [1].

KCl in the presence of nigericin, or K-acetate in the presence of valinomycin, where charge and proton balance during osmotic swelling required the translocation of protons across the membrane. It was now found that albumin and nucleotide both restricted a high basal proton permeability and that the two were synergistic [12] correlating with the induction of respiratory control. This was probably the first demonstration in mitochondria that a native proton conductance (rather than one induced by a synthetic uncoupler [13]) was able to control respiration. However passive swelling experiments generated low diffusion potentials rather than the high proton electrochemical potential ($\Delta\mu_{H+}$) postulated by Mitchell and shown for liver mitochondria in his classic 1969 paper [14], where he and Jennifer Moyle used pH and K^+-selective electrodes to calculate the pH and $\Delta\psi$ components of $\Delta\mu_{H+}$ in a low K^+ medium in the presence of valinomycin to allow the K^+ to equilibrate according to the Nernst potential.

A predilection for simplistic models, and a belated in-depth reading of Mitchell's reviews, led me to realize that the proton circuit could be considered to be analogous to a simple electrical circuit, with the proton-translocating respiratory chain complexes as electrical 'batteries', the membrane potential (or strictly $\Delta\mu_{H+}$), as the voltage term, the respiratory rate multiplied by the H/O stoichiometry giving the proton current, and the total resistance of the proton re-entry pathways (or rather the reciprocal conductance) determined by Ohm's Law (Fig. 2). This concept, which continues to guide our research 35 years later, suggested that parallel monitoring of $\Delta\mu_{H+}$ and respiration could allow me to quantify the proton conductance of BATM under respiring conditions. One problem was that Mitchell's technique [14], while ingenious, was labor intensive and technically demanding. Using rat liver mitochondria to establish the methodology, I modified Mitchell's electrode technique into an isotopic one, in which the distribution of ^{86}Rb (in the presence of valinomycin), ^{14}C-methylamine and ^{3}H-acetate allowed the two components of $\Delta\mu_{H+}$ to be rapidly and sensitively determined [3]. The value obtained for $\Delta\mu_{H+}$ with succinate as substrate (228 mV in state 4 and 170 mV in state 3, taking Mitchell's value for the matrix volume) was close to the Mitchell value. We thus had a high-throughput method for determining $\Delta\mu_{H+}$ for isolated mitochondria, and hence the voltage term in the proton circuit. Proton current was estimated from the rate of respiration in parallel experiments and Ohm's Law allowed me to calculate the proton conductance of the inner membrane (C_mH^+) in nmol $H^+/min^{-1}/mg^{-1}$ mV^{-1} [3,4]. One finding in this initial study was that I could determine the current/voltage relationship of the basal proton leak by titrating down succinate respiration with malonate. C_mH^+ decreased rapidly below 200 mV — the first description of the 'non-ohmic' proton leak. I subsequently refined the technique to monitor the current/voltage relationships for uncoupling protein 1 (UCP1) in the presence an absence of its acute activator, fatty acid [15], see Fig. 3B. This approach has subsequently been developed in a number of laboratories, notably that of Brand [16,17].

Returning to the BATM proton leak, I found that the freshly prepared mitochondria had an enormous proton conductance and were incapable of maintaining more than a few mV of protonmotive force [4], but that albumin, purine nucleotides and pH controlled the C_mH^+ over more than a 30-fold range. Excitingly, a parallel experiment monitoring the effects of the agents on proton-dependent passive swelling closely reproduced these dependencies. This was of major

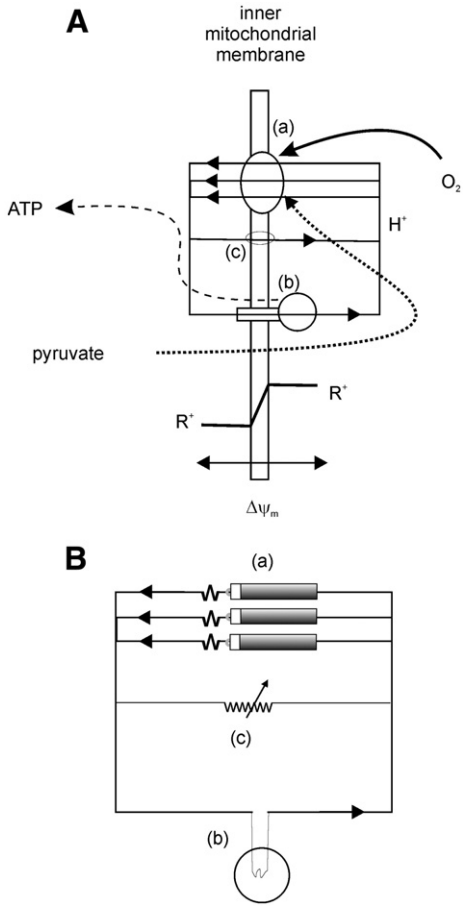

Fig. 2. The proton circuit A. Simplified representation of the mitochondrial proton circuit. (a) respiratory chain; (b) ATP synthase; (c) proton leak pathway (UCP). The accumulation of a lipophilic cation R^+ across the inner membrane is shown as the generic means to estimate $\Delta\psi_m$ B. Simplistic representation of the 'equivalent' electrical circuit: (a) three batteries in parallel representing the proton-translocating complexes. Note that each has an internal resistance such that the voltage falls as the current drawn increases, mimicking the drop in $\Delta\mu_{H+}$ as the proton current increases; (b) light bulb as the ATP synthase analogue; (c) variable resistance 'short circuit' mimicking proton leak pathways.

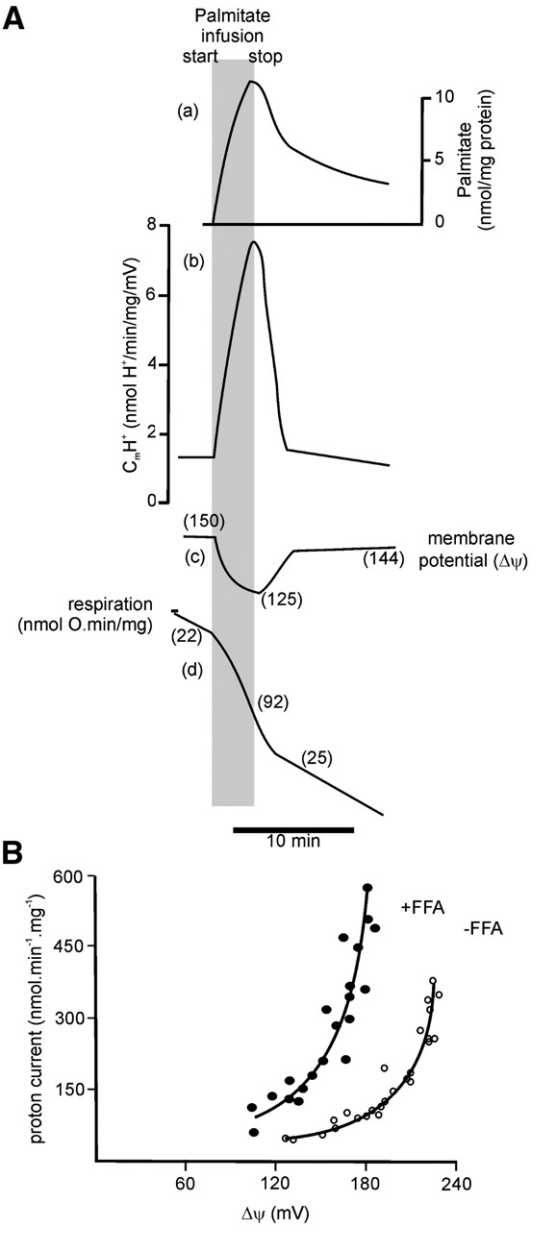

Fig. 3. UCP1 control of respiration modeled in isolated brown adipose tissue mitochondria (BATM). A Mitochondria were incubated in a combined oxygen/tetraphenylphosphonium electrode chamber to monitor respiration and Δψ. CoA, ATP and L-carnitine were present to allow fatty acid activation and the initial (non-thermogenic) substrate was pyruvate. For the period indicated [^{3}H]-palmitate was infused into the incubation, mimicking lipolysis. As the concentration of palmitate rose, activating UCP1 (a) the proton conductance (C_mH^+) increased (b), lowering Δψ (c) and allowing respiration to increase (d). When the infusion was terminated, residual palmitate was activated and oxidized (a), leading to a fall in C_mH^+ (b), mitochondrial repolarization (c) and respiratory inhibition as the mitochondria reverted to pyruvate oxidation. From [15]. B. Current/voltage relationships for the proton leak in BATM in the presence of purine nucleotide and in the absence (open points) and presence (closed points) of 1.3 μM unbound palmitate. The titration was produced by infusing palmitoyl-L-carnitine at decreasing rates and demonstrates that the activating 'messenger' fatty acid acts by lowering the level of Δψ at which conductance increases. Data from [15].

significance, since it showed that 'uncoupling' could be equated with proton leak in a system with an inhibited respiratory chain and in the absence of synthetic uncouplers, thus invalidating the alternative that uncoupling was due some 'slip' in energy transduction in the respiratory chain (reviewed in [18]). The beautiful simplicity of BATM bioenergetics (for example a major substrate, α-glycerophosphate, was oxidized at the outer face of the inner membrane, removing complications of prior transport into the matrix) allowed the proton circuit model to be consolidated. For example, in addition to establishing the current/voltage relationships of the mysterious fatty acid activated and GDP-inhibited proton leak in these mitochondria [15], the feed-back inhibition exerted by $\Delta\mu_{H+}$ on electron transport down the respiratory chain activity could be quantified [4,19]. In these papers it was apparent that a slight decrease in $\Delta\mu_{H+}$ could produce a large increase in respiration. In the electrical circuit analogy this meant that the 'internal resistance' of the 'batteries' (Fig. 2B) corresponding to the proton-translocating complexes was low. This finding has been the basis of our critique [20] of recent 'mild-uncoupling' hypotheses [21–25], which propose that an increase in C_mH^+ could be a valid method for decreasing $\Delta\mu_{H+}$ and hence the production of reactive oxygen species, see also [26]. In other words, the energetic cost of diverting the proton current into a dissipative pathway and hence decreasing the capacity for ATP production, outweighed any gain by alleviating oxidative stress [20].

The brown fat studies in which we established the nature of the purine nucleotide binding site [27]and identified UCP1 [28] have been recently reviewed [29,30] and will not be recapitulated here. However, the current model for the fatty acid control of the uncoupling protein in intact brown adipocytes [31] is a direct application of the proton circuit approach and will be briefly summarized. In the intact brown adipocyte β-adrenergic stimulation of lipolysis delivers fatty acids to the mitochondrion (reviewed in [32]). Our hypothesis [31] was that fatty acid not only acts as the substrate for thermogenesis, but is also the activator of UCP1. For this to be self-regulating the affinity of acyl CoA synthetase for fatty acid must be sufficient to pull the 'uncoupling' fatty acid off the uncoupling protein at the termination of lipolysis to 'recouple' the mitochondrion, end thermogenesis, and resume the basal, pyruvate supported, respiration of the mitochondria. To test this, Rebecca Locke, Eduardo Rial and I incubated BATM in the presence of pyruvate, CoA, carnitine and ATP with parallel measurement of respiration and Δψ [31]. A slow infusion of [^{3}H] palmitate then mimicked lipolysis. As the concentration of palmitate increased (Fig. 3A), the C_mH^+ of UCP1 increased in parallel. This in turn lowered Δψ and allowed respiration (fed by the generated fatty acyl carnitine) to increase. Terminating infusion reversed each process as the residual palmitate was oxidized and the fatty acid dissociated from UCP1.

The insight given by the BATM model facilitated our investigations into the next area — the regulation of the mitochondrial Ca^{2+} circuit.

2. The linkage of the mitochondrial H^+, Na^+ and Ca^{2+} circuits

While the chemiosmotic theory beautifully explained mitochondrial respiration and ATP synthesis, Ca^{2+} transport was problematical. If the uptake of Ca^{2+} into the matrix was electrophoretic in response to the membrane potential, the Nernst equation predicted that, as a divalent cation, the equilibrium concentration gradient of Ca^{2+} across the inner membrane could be as high as 10^5. This dilemma was resolved by the finding by Crompton and colleagues [33,34] of an independent Na^+/Ca^{2+} exchanger in the inner membrane of a variety of mitochondria, and the description of Na^+ and Ca^{2+} cycles interlocking with the primary proton circuit (Fig. 4)consistent with the original schematic circuit described by Mitchell in 1966 (Fig. 1). Using a Ca^{2+}-selective external electrode, we determined that liver and brain mitochondria could reduce the external free Ca^{2+} concentration to between 0.5 and 1 μM and that this value was largely independent of matrix Ca^{2+} load over a wide range, as long as phosphate was present in excess as a counterion [5,35], Fig. 5C. In order to relate this behaviour to the apparently symmetrical ion cycling across the inner membrane (Fig. 4) it was necessary to define the kinetics of both the uptake (uniporter) and efflux (Na^+/Ca^{2+} exchange) pathways.

The activity of the efflux pathway in both brain and liver mitochondria (the latter having a $2H^+/Ca^{2+}$ rather than a Na^+/Ca^{2+} activity) turned out to be controlled by the free matrix Ca^{2+} concentration, which was defined by the solubility product of the $Ca_3(PO_4)_2$ complex in the matrix [36,37]. This

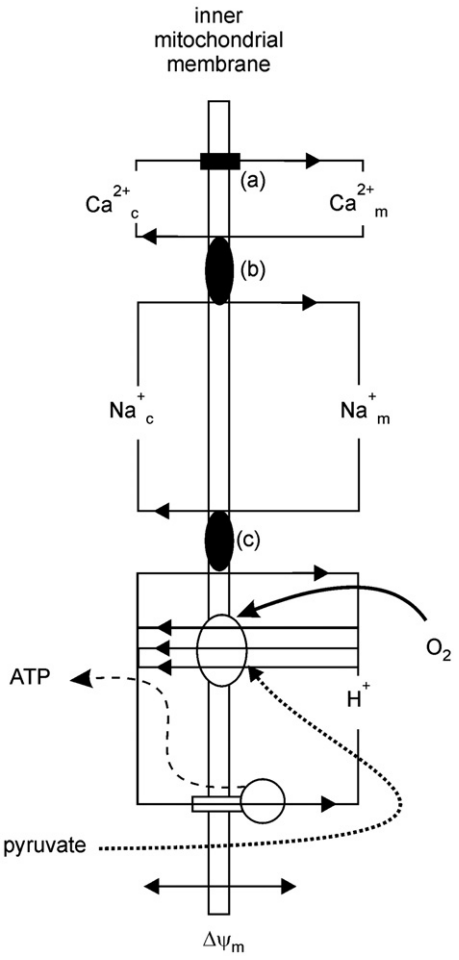

Fig. 4. The coupling of the mitochondrial proton and calcium circuits. The Ca^{2+} uniporter (a) is coupled to the proton circuit in heart and brain mitochondria via a Na^+/Ca^{2+} exchanger (b) and a Na^+/H^+ exchanger (c).

in turn was determined by the matrix PO_4^{3-} concentration which was finally dependent on the external phosphate concentration and the pH gradient across the inner membrane [37]. This chain of events meant that as long as the matrix contained sufficient Ca^{2+} for the $Ca_3(PO_4)_2$ complex to form (>10 nmol Ca^{2+}/mg protein), mitochondria respiring in the presence of excess phosphate showed a constant Ca^{2+} efflux rate over a range from 10 to >500 nmol Ca^{2+}/mg protein [36].

The activity of the Ca^{2+} uniporter as a function of external free Ca^{2+} concentration ($[Ca^{2+}]_e$) was determined under these conditions by infusing the cation at a slow constant rate and determining the value at which $[Ca^{2+}]_e$ reached a steady state, meaning that the net rate of Ca^{2+} uptake into the matrix (the difference between the uniporter and Na^+/Ca^{2+} activities) exactly equaled the rate of infusion [36]. By varying the rate of infusion the dependency of the uniporter on $[Ca^{2+}]_e$ could be determined, and it was found that its activity in liver mitochondria increased as the 2.5 power of $[Ca^{2+}]_e$ in the range from 1 to 4 μM [36]. In this buffering mode, therefore, mitochondria would be predicted to seek to oppose an elevation in $[Ca^{2+}]_c$ above the level at which uptake and efflux balance (the 'set-point') by temporarily accumulating Ca^{2+}, releasing it when the plasma membrane Ca^{2+} pumps lower $[Ca^{2+}]_c$ below this value (Fig. 5B). Several studies with intact cultured cells have confirmed this prediction [38–40].

An apparent discrepancy between this Ca^{2+} buffering mode and the convincing evidence that changes in matrix free Ca^{2+} can regulate the activity of key tricarboxylic acid cycle enzymes [41,42] was resolved when it was demonstrated that mitochondria switch seamlessly between the 'matrix regulation' and 'buffering' modes when sufficient Ca^{2+} has accumulated in the matrix to initiate formation of the $Ca_3(PO_4)_2$ complex [37], Fig. 5A.

3. The mitochondrial proton circuit in the isolated nerve terminal and cultured neuron

While the emphasis in the UCP and Ca^{2+} studies with isolated mitochondria was to devise conditions that were as close as possible to those pertaining in the intact cell, the isolated mitochondrial milieu will inevitably deviate from the *in situ* cytoplasm, while interactions with the plasma membrane will be absent. In an attempt to bridge the gap between isolated mitochondria and the considerable complexity

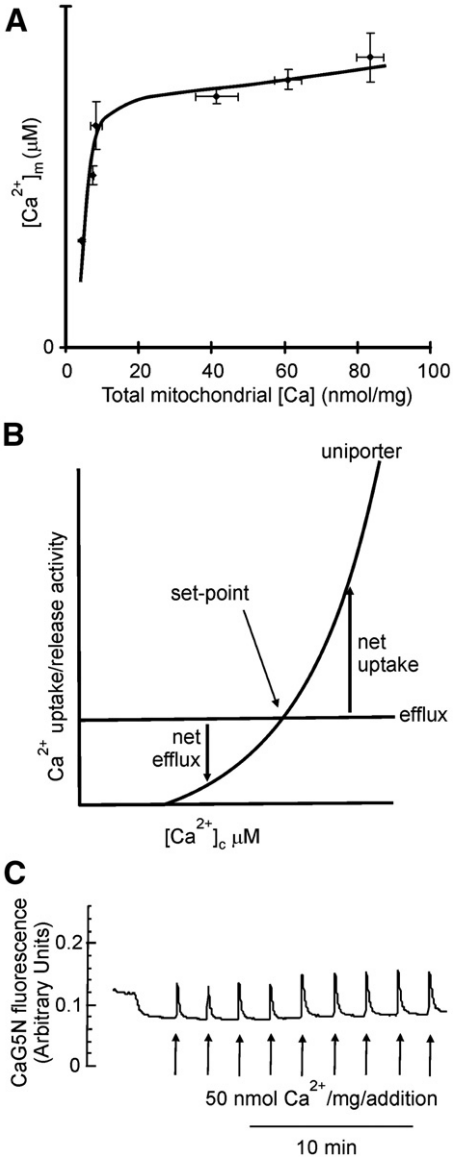

Fig. 5. The regulation of matrix and cytoplasmic free Ca^{2+} concentrations by the calcium circuit. A The formation of a $Ca_3(PO_4)_2$ complex in the matrix buffers the free matrix Ca^{2+} ($[Ca^{2+}]_m$) so that it is essentially independent of total matrix Ca^{2+} when this is above 10 nmol mg^{-1}. Below this loading $[Ca^{2+}]_m$ varies with load allowing matrix dehydrogenases to be regulated [37]. B. The constant $[Ca^{2+}]_m$ means that the activity of the Na^+/Ca^{2+} exchanger is independent of total matrix Ca^{2+} over the same range [36]. In contrast, the activity of the Ca^{2+} uniporter increases sharply with cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) [36]. The set-point at which uptake and efflux balance is in the region of 0.5 μM and is independent of total matrix Ca^{2+} over a wide range. C. The kinetics of the two pathways explain why the external free Ca^{2+} returns to the same value (set-point) after multiple Ca^{2+} additions (from [37]).

of the intact cell, the isolated nerve terminal, or synaptosome, was chosen as an initial model. Axonal varicosities rapidly reseal after homogenization of brain regions, producing 2 μm diameter synaptosomes that retain functional mitochondria, a largely intact cytoplasm and a plasma membrane that possesses the full range of ion pumps, channels, receptors and exo- and endocytic machinery characteristic of the intact terminal (for review see [43]). At the same time the synaptosomes can be manipulated in much the same way as isolated mitochondria, for example measuring respiration in conventional oxygen electrode chambers, and furthermore possess a significant advantage over primary cell cultures that the preparation can readily be made from brains of animals of any age.

The linkage between the mitochondrial proton and calcium circuits and the major plasma membrane ion circuits is shown in Fig. 6. Analysis of the mitochondrial proton circuit in a synaptosome or intact neuron, as with isolated mitochondria, requires the separate monitoring of proton current (mitochondrially-coupled respiration) and mitochondrial membrane potential ($\Delta\psi_m$). In most cells the large majority of the oxygen uptake is mitochondrial, and non-mitochondrial respiration can be determined by the residual respiration in the presence of electron transport inhibitors such as rotenone plus myxothiazol. The basal mitochondrial respiration reflects the proton current driving ATP synthesis, plus that utilized by the inherent inner membrane proton leak. Addition of oligomycin allows the leak to be quantified, giving the proton current driving ATP synthesis by difference (ignoring any increase in C_mH^+ accompanying the hyperpolarization after the inhibitor). The third important parameter in the context of neuronal survival is the 'spare respiratory capacity' (SRC), defined as the maximal increase in respiration above basal attained in the presence of a carefully titrated concentration of protonophore. For isolated brain mitochondria the maximal respiration obtained in State 3 (in the presence of ADP) is close to that in the presence of protonophore, indicating the ATP synthesis and export has sufficient capacity to utilize the maximal proton current generated by the respiratory chain. The SRC is thus a measure of the ATP generating reserve available to cope with an increase in ATP demand, for example by the Na^+/K^+-ATPase and Ca^{2+}-ATPase at the plasma membrane as they restore ionic homeostasis following channel or receptor activation.

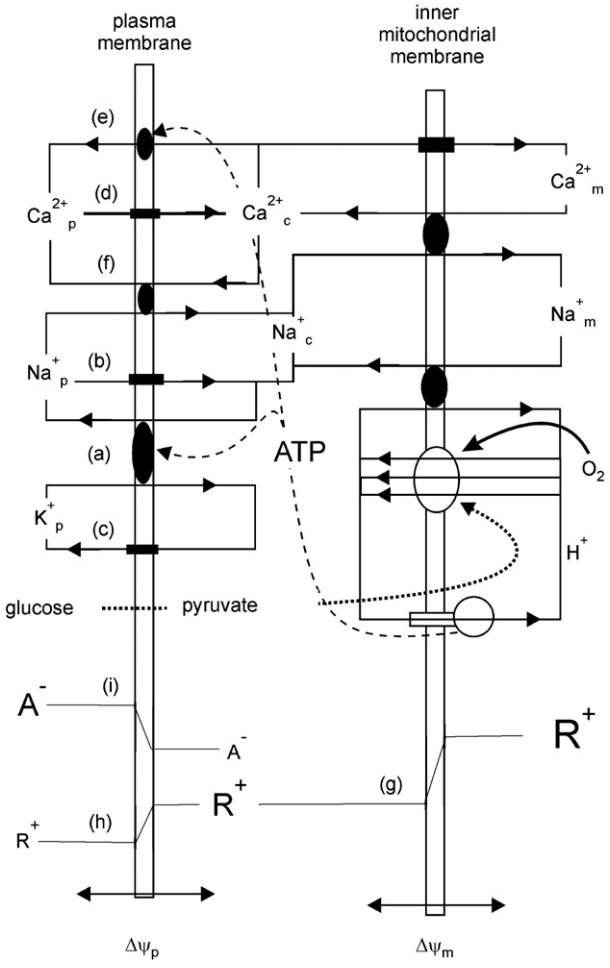

Fig. 6. The linkage of the mitochondrial proton circuit to plasma membrane ion circuits in an intact neuron. The Na^+/K^+-ATPase (a) cycles Na^+ and K^+ across the membrane although the low basal Na^+ conductance (b) means that ion cycling, and hence ATP utilization, is low. The high constitutive K^+ conductance (c) means that the plasma membrane potential ($\Delta\psi_p$) is close to the K^+ equilibrium potential. Activating Na^+ entry via voltage-activated channels or receptors can greatly enhance ion cycling and hence ATP utilization by the Na^+/K^+-ATPase. Ca^{2+} cycles across the plasma membrane, entering via channels and receptors (d) and being extruded by a Ca^{2+}-ATPase (e) or by Na^+/Ca^{2+} exchange (f). When $[Ca^{2+}]_c$ rises above about 0.5 μM there is net accumulation into the matrix (see Fig. 3). $\Delta\psi_m$ can be monitored by the concentration gradient of a fluorescent cation between cytoplasm and matrix (g) or by the total accumulation relative to the external medium (h) in which case the contribution of the plasma membrane potential (i) must be taken into account and can be quantified by the distribution of a lipophilic anion.

SRC has proven to be a key factor in the ability of neurons to survive chronic exposure to the neurotransmitter glutamate in models of the excitotoxicity accompanying stroke. The failure of mitochondrial ATP synthesis in ischemic tissue leads to a collapse in the Na^+ gradient across the plasma membrane, reversal of the Na^+-coupled glutamate re-uptake carriers and a massive increase in extracellular glutamate which diffuses out of the ischemic core of the infarct to cause chronic activation of NMDA-selective glutamate receptors on neurons in the surrounding penumbra (reviewed in [44]). NMDA receptors do not desensitize in the continued present of glutamate and cause the massive uptake of Na^+ and Ca^{2+} into the cytoplasm. The life-or-death struggle of the neuron will be decided by the ability of the mitochondrion to accumulate and retain Ca^{2+} without undergoing the permeability transition, and by the capacities of the Ca^{2+}- and Na^+/K^+-ATPases to extrude the ions flooding into the cell. The greatly increased ATP demand by the ion pumps together with the additional demand on the proton circuit driving net accumulation of Ca^{2+} into the matrix makes it apparent that glutamate exposure can place a tremendous bioenergetic load upon the intra-neuronal mitochondria, that can be quantified by monitoring the respiration of cultured neurons exposed to glutamate.

The technical problems inherent in measuring the respiration of a 2-dimensional array of cells attached to a substrate was solved some years ago by our development of the cell respirometer [8], in which medium is slowly superfused over the neurons in a thin closed imaging chamber and the downstream oxygen deficit is determined by a flow-through micro-oxygen electrode. Parallel confocal imaging of a field allows the state of the neurons to be monitored with fluorescent probes. Depending on the age of the neurons in culture, glutamate exposure can utilize virtually the full respiratory capacity of the mitochondria [45]. An example of this is shown in Fig. 7A. Rat cerebellar granule neurons were superfused with glutamate and respiration increased to 300% of control. After perfusing away the amino acid an optimal concentration of FCCP was added and respiration increased to the same extent, indicating that NMDA receptor activation was utilizing the full spare respiratory capacity of the *in situ* mitochondria [45]. Any manipulation that decreases the SRC increases cell death in the presence of glutamate. This includes mitochondrial manipulations where the emphasis in the literature has been upon the generation or suppression of reactive oxygen species, e.g. titration with low rotenone concentrations to enhance ROS levels [46] or titration with low protonophore concentrations in an attempt to lower $\Delta\psi_m$ and decrease ROS [47]. Both treatments compromise the SRC to the extent that demand exceeds supply, as monitored in the cell respirometer, and both greatly potentiate excitotoxic cell death [20,45].

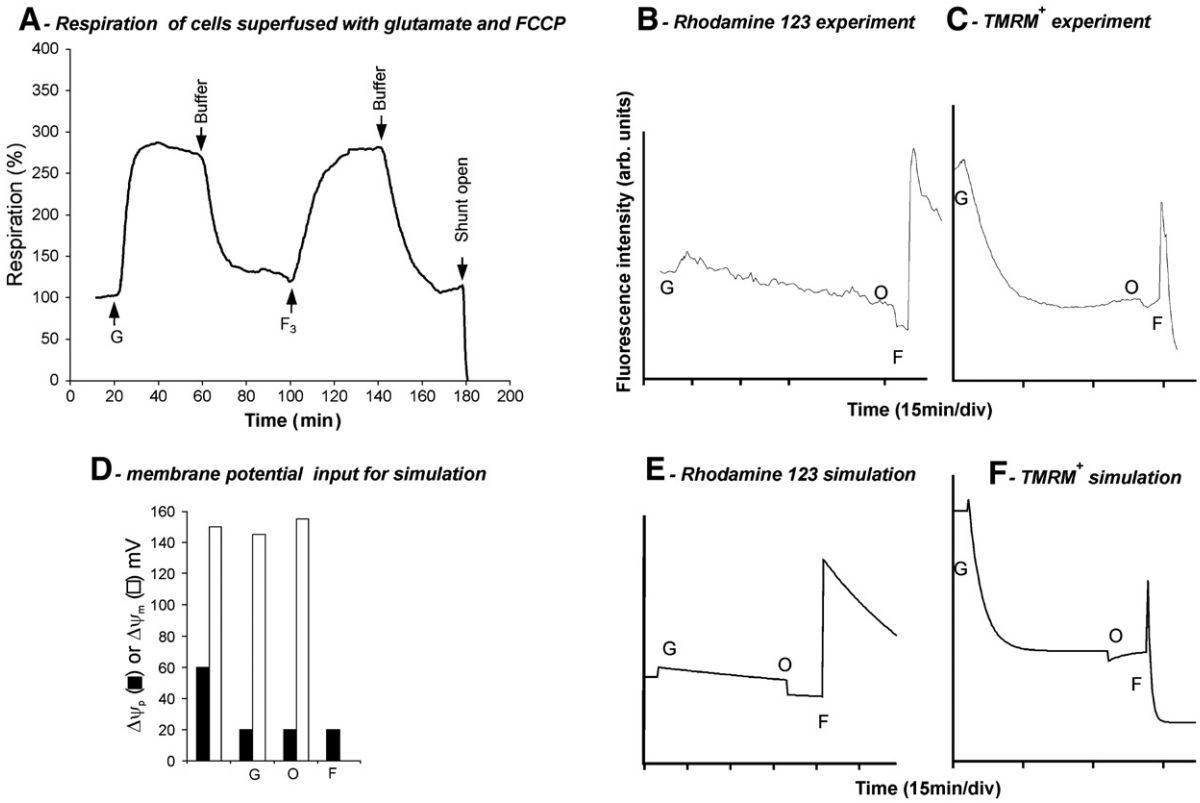

Fig. 7. Membrane potential and respiration of *in situ* granule cell mitochondria exposed to glutamate. *(A)* respiration of granule neurons superfused with glutamate (G) and 3 μM FCCP (F_3). Data from [45]. *(B,C)* Single cell fluorescence of a rat cerebellar granule neuron loaded with rhodamine 123 or 50 nM $TMRM^+$. Additions were made of 100 μM glutamate plus 10 μM glycine (G), 2 μg/ml oligomycin (O) and 1 μM FCCP (F). *(D)* plasma and mitochondrial membrane potentials input into the simulation [7] to produce the simulated traces (*E,F, E*). identical parameters were used for both simulations except that the rate constant for equilibration of $TMRM^+$ across the plasma membrane was 20-fold higher than for rhodamine 123. Data from [7].

The voltage term ($\Delta\psi_m$) in the proton circuit must not be forgotten in this context. Membrane-permeant fluorescent cations have been used to detect qualitative changes in $\Delta\psi_m$ for several years [48], although their use had generally been restricted to a yes–no answer to the question 'are the mitochondria polarized or depolarized?' In 2000 we put the use of the commonly employed probes TMRM and rhodamine 123 on a more quantitative basis by considering the biophysical basis of the single-cell signal [7]. Three simple assumptions were found to be sufficient to predict and describe the whole-cell fluorescent responses to a change in $\Delta\psi_p$ and/or $\Delta\psi_m$. Firstly that the probe seeks to attain a Nernst equilibrium across both the plasma and mitochondrial membranes; secondly that equilibration across the small highly invaginated inner mitochondrial membrane was much more rapid than across the cell body's plasma membrane reflecting the differing surface-to-volume ratios; and finally that the probes aggregated and became non-fluorescent within the matrix at a critical concentration. The analysis of the traces proved to be highly dependent upon whether this aggregation concentration was exceeded ('quench mode') or whether the probe remained monomeric and fluorescent within the matrix (non-quench-mode) [7].

This simple analysis proved to be particularly useful in quench mode for the interpretation of complex fluorescence traces in case where changes were occurring in both $\Delta\psi_p$ and $\Delta\psi_m$, for example when the NMDA-selective glutamate receptor was activated in order to model the consequences of excitotoxic exposure of neurons to glutamate in the aftermath of stroke (for review see [49,50]). Fig. 7B, C shows single-cell fluorescence traces for the same experiment performed in duplicate with the permeant $TMRM^+$ and the 20-fold slower equilibrating rhodamine 123. The actual experiment involved the addition of glutamate to activate NMDA receptors, followed by oligomycin to inhibit the ATP synthase and FCCP to collapse $\Delta\psi_m$. The computer simulations (Fig. 7E, F) were generated by inputting the same indicated changes in membrane potentials (Fig. 7D) with the only difference that the rate constant for permeation of rhodamine 123 across the plasma membrane was set 20-fold slower than for $TMRM^+$ [7]. This experiment reveals several important features about the initial response of the intra-neuronal mitochondria to the massive influx of Ca^{2+} and Na^+ into the cytoplasm through the pathophysiologically activated NMDA receptors. First, the difference in re-equilibration rates across the mitochondrial and plasma membranes means that the slight mitochondrial depolarization on receptor activation (seen as the transient increase in fluorescence due to de-quenching of the probe as it leaves the mitochondria) can be separated in time from the slow decrease in signal as the probe leaves the cell due to the plasma membrane depolarization. Also included in these traces is the 'oligomycin null-point test' to establish whether the mitochondria are still able to generate ATP in the presence of glutamate. Thus if oligomycin causes a mitochondrial hyperpolarization then this indicates that ATP synthesis was occurring prior to inhibitor addition, whereas a depolarization indicates that $\Delta\psi_m$ was being maintained by ATP synthase reversal utilizing cytoplasmic ATP. In the indicated experiment, hyperpolarization (increased quenching) shows that the mitochondria remained bioenergetically competent in the face of this chronic receptor activation. Indeed when the potentials are input into the simulation to reproduce the experimental traces, $\Delta\psi_m$ drops by only about 5 mV with glutamate, while the subsequent oligomycin-induced hyperpolarization was estimated to be 10 mV [7]. Recently, we refined the membrane potential technique to include a fluorescent membrane-permeant anion to allow simultaneous direct monitoring of changes in plasma membrane potential, $\Delta\psi_p$ [51].

4. Conclusion

The revolutionary scheme proposed by Peter Mitchell in 1966 and reproduced here in Fig. 1 has turned out to precisely fit with our current understanding of the ion transport pathways across the inner mitochondrial membrane, and to provide such a fertile field for experimentation that it has provided the basis for many research careers. Judged by the criteria that a pre-eminent theory should combine elegance, simplicity and prediction, the proton circuit component of the chemiosmotic hypothesis has survived forty years of experimental testing unchanged, whereas Mitchell's proposals for the molecular mechanisms of the proton pumps have had to be drastically revised in the light of modern structural and functional protein chemistry.

Acknowledgements

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